Volume 165, number 2 FEBS 1108 January 1984

A comparison of the phosphorylated and unphosphorylated forms of isocitrate dehydrogenase from *Escherichia coli* ML308

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Received 23 November 1983

$NADP^+$ can protect active isocitrate dehydrogenase against attack by several proteases. Inactive phosphorylated isocitrate dehydrogenase is much less susceptible to proteolysis than the active enzyme, and it is not protected by $NADP^+$. The results suggest that binding of $NADP^+$ to, or phosphorylation of, active isocitrate dehydrogenase induces similar conformational states. Fluorescence titration experiments show that NADPH can bind to active but not to inactive isocitrate dehydrogenase. It is suggested that the phosphorylation of isocitrate dehydrogenase may occur close to its coenzyme binding site.

Isocitrate dehydrogenase *Protein phosphorylation* *NADP* *Binding study* *Proteolysis* *Glyoxylate cycle*

1. INTRODUCTION

The NADP-linked isocitrate dehydrogenase (ICDH) of *Escherichia coli* and *Salmonella typhimurium* was the first bacterial enzyme shown to be regulated by phosphorylation/dephosphorylation [1,2], reviewed in [3], The phosphorylation system, which involves a specific bifunctional ICDH kinase/phosphatase [4,5] is thought to be involved in controlling carbon flux between the Krebs cycle and the glyoxylate bypass during growth on acetate [3,6]. We have recently put forward more detailed proposals concerning the role and control of the phosphorylation of ICDH in vivo [7].

ICDH is essentially totally inactivated by phosphorylation of a single serine residue per subunit [5,8]. We have investigated the differences between the active and inactive forms of ICDH by limited proteolysis and by fluorimetric binding studies. The results indicate that the inactive form of ICDH is unable to bind NADP.

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2. EXPERIMENTAL

2.1. *Materials*

Trypsin and chymotrypsin were obtained from Worthington, protease K, thermolysin and NADPH from Boehringer, subtilisin Carlsberg from Sigma and *S. aureus* V8 protease from Miles. The sources of other chemicals were as in [9]. Active ICDH was isolated from *E. coli* ML308 as in [9] and ^{32}P-labelled inactive ICDH was prepared as in [5]. This material is referred to as inactive ICDH.

2.2. *Proteolysis experiments*

Samples of ICDH were incubated with proteases as described in the text. The release of ^{32}P-labelled trichloroacetic acid soluble material from inactive ICDH was estimated by the addition of trichloroacetic acid to 5% (w/v); bovine serum albumin (20 mg/ml) was present as a carrier. After centrifugation, portions of the supernatant were

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counted as in [5]. The remaining trichloroacetic acid precipitable ^{32}P is expressed as a percentage of the total ^{32}P present at zero time; no ^{32}P-release was detectable in the absence of protease. ICDH activity was assayed as in [9]. To quantitate degradation of the 45-kDa protein that corresponds to ICDH [9] samples were denatured and analyzed on 15% polyacrylamide gels [10] and the gels were scanned with an LKB laser densitometer. For the latter two sets of measurements results are expressed as percentages of the zero time value.

2.3. *Fluorescence titration experiments*

Samples of ICDH were dialysed exhaustively into 50 mM Mops–NaOH, 1 mM EDTA, 1 mM dithiothreitol (pH 7.3). This completely freed the enzyme of $NADP^+$; addition of 2.5 mM DL-isocitrate and 1.5 mM $MnCl_2$ to a solution of the enzyme (5 μM in terms of subunits) generated no NADPH as judged by fluorescence measurements.

Fluorescence was measured at 25°C using a Hitachi Perkin-Elmer MPF 2A spectrofluorimeter; excitation and emission were at 340 nm and 460 nm, respectively. Titration experiments were carried out by adding successive small aliquots of an NADPH solution (0.931 mM) to cuvettes containing ICDH (0.225 mg/ml, 5 μM in terms of subunits) in the above buffer. Protein concentrations were determined by absorbance measurements at 280 nm using the absorbance index for homogeneous ICDH given in [9].

3. RESULTS

Table 1 shows the results of an experiment in which the active and inactive forms of ICDH were incubated with chymotrypsin for a fixed period. For both forms of the enzyme proteolysis was assessed by densitometric scanning of the 45-kDa band after gel electrophoresis; we also measured the decrease in activity of active ICDH and the release of trichloroacetic acid-soluble radioactivity from inactive ICDH.

The results reveal two interesting points. Firstly, $NADP^+$ significantly protects active ICDH against proteolysis, judged both by loss of activity and by loss of the 45-kDa band. The concentration of $NADP^+$ used in this experiment (0.35 mM) is

Table 1

Digestion of ICDH by chymotrypsin

	Active ICDH		Inactive ICDH	
Protein: protease	Activity remaining	Protein remaining	^{32}P remaining	Protein remaining
		$NADP^+$ absent		
36:1	22	19	96	92
72:1	28	30	97	91
183:1	35	37	99	97
367:1	45	46	100	91
		$NADP^+$ present		
36:1	74	67	98	83
72:1	82	74	99	81
183:1	75	68	100	91
367:1	83	67	100	93

Samples of ICDH (0.22 mg/ml) were digested with the indicated protein:protease ratios (by wt) for 15 h at 37°C. Figures in the 'Protein remaining' column refer to the amount of 45-kDa band that corresponds to pure ICDH, determined densitometrically. Figures in the '^{32}P remaining' column refer to the percentage of ^{32}P that was trichloroacetic acid precipitable. Active ICDH was digested in 44 mM Mops–NaOH, 0.9 mM EDTA (pH 7.3), 9.6% (v/v) glycerol. Phosphorylated ICDH was digested in 35 mM Mops–NaOH, 0.5 mM EDTA (pH 7.3), 10% (v/v) glycerol. $NADP^+$ was used at 0.35 mM. Values are expressed as %

20–30 times greater than the K_m of ICDH for $NADP^+$ at 0.05 mM DL-isocitrate (unpublished). Secondly, the inactive form of ICDH is almost completely resistant to proteolysis by chymotrypsin. In this experiment the incubation conditions used for the two forms of ICDH were slightly different (see table 1) but it seems clear that phosphorylation significantly protects ICDH against proteolysis.

We also investigated proteolysis of active ICDH using protease K, subtilisin, trypsin, thermolysin and *S. aureus* V8 protease; in each case $NADP^+$ protected the enzyme against loss of activity (not shown). The effects of V8 protease on the active and inactive forms of ICDH were investigated fully. The results (table 2) show that $NADP^+$ protected active ICDH in the presence or absence of Mn^{2+}, which is required for ICDH activity; Mn^{2+} alone protected the enzyme to some extent. The results also show that V8 protease was more effective than chymotrypsin in attacking inactive ICDH. It is again clear that the inactive form of ICDH is much less susceptible to proteolysis than is the active form, and the data suggest that $NADP^+$ is unable to protect the inactive enzyme, although Mn^{2+} can do so.

In other experiments we have found that 2.5 mM DL-isocitrate can protect both active and inactive forms of ICDH against proteolysis by V8 protease (not shown). We have also investigated whether proteolysis of inactive ICDH could cause reactivation of the enzyme. In a series of experiments with protease K, V8 protease, trypsin, chymotrypsin and subtilisin in which 3–100% of the ^{32}P-radioactivity was rendered trichloroacetic acid-soluble, we were unable to detect any reactivation of the enzyme.

We have also assessed the effects of $NADP^+$ on the proteolysis of the two forms of ICDH in time-course experiments. The results shown in fig.1 con-

Table 2

Digestion of ICDH by *S. aureus* V8 protease

	Active ICDH		Inactive ICDH	
Protein: protease	Activity remaining	Protein remaining	^{32}P remaining	Protein remaining
	$NADP^+$ absent, Mn^{2+} absent			
22:1	16	17	71	47
44:1	25	27	83	68
88:1	46	52	90	82
176:1	80	99	95	79
	$NADP^+$ present, Mn^{2+} absent			
22:1	73	56	80	50
44:1	83	63	90	77
88:1	98	80	94	87
176:1	100	83	96	80
	$NADP^+$ absent, Mn^{2+} present			
22:1	38	58	90	94
44:1	66	90	95	100
	$NADP^+$ present, Mn^{2+} present			
22:1	73	86	95	99
44:1	86	100	97	91

Samples of ICDH (0.24 mg/ml) were digested with the indicated protein:protease ratios (by wt) for 17 h at 37°C. Digestion was carried out in 50 mM Mops-NaOH, 1 mM EDTA (pH 7.3), 5% (v/v) glycerol in the presence or absence of $NADP^+$ (0.35 mM) or $MnCl_2$ (1.3 mM). Values are expressed as %

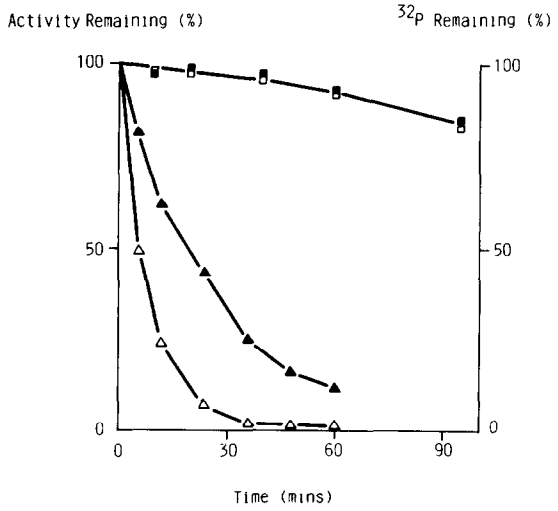

Fig.1. Digestion of ICDH by *S. aureus* V8 protease. ICDH (0.24 mg/ml) was digested with V8 protease (0.1 mg/ml) in 30 mM Mops–NaOH, 1 mM EDTA (pH 7.3), 5% (v/v) glycerol, in the presence (▲,■) or absence (△,□) of 0.4 mM $NADP^+$. Digestion of active ICDH (△,▲) was monitored by loss of ICDH activity and that of phosphorylated ICDH (□,■) by release of trichloroacetic acid-soluble radioactivity.

firm that, under identical conditions, $NADP^+$ protects the active form but not the inactive form against attack by V8 protease. The loss of activity of active ICDH followed pseudo-first order kinetics; saturating levels of $NADP^+$ reduced the rate constant for inactivation by a factor of about 4 (not shown). Phosphorylation of ICDH reduced the rate of proteolysis by a much greater factor.

The correlation between the loss of activity and the destruction of the 45-kDa component for active ICDH is shown in fig.2a. The data clearly fall into two distinct groups corresponding to the presence or absence of $NADP^+$. The data from experiments in the absence of $NADP^+$ fall close to a line of slope, approximately one that passes near to the origin; there is thus an almost exact correlation between loss of activity and loss of the 45-kDa component that corresponds to the intact ICDH subunit. The data from experiments in the presence of $NADP^+$ fall close to a different line, displaced upwards from the first line. Our interpretation of this result is that $NADP^+$ can stabilise an active 'nicked' form of ICDH against further proteolysis; such a species is not stable in the absence of $NADP^+$.

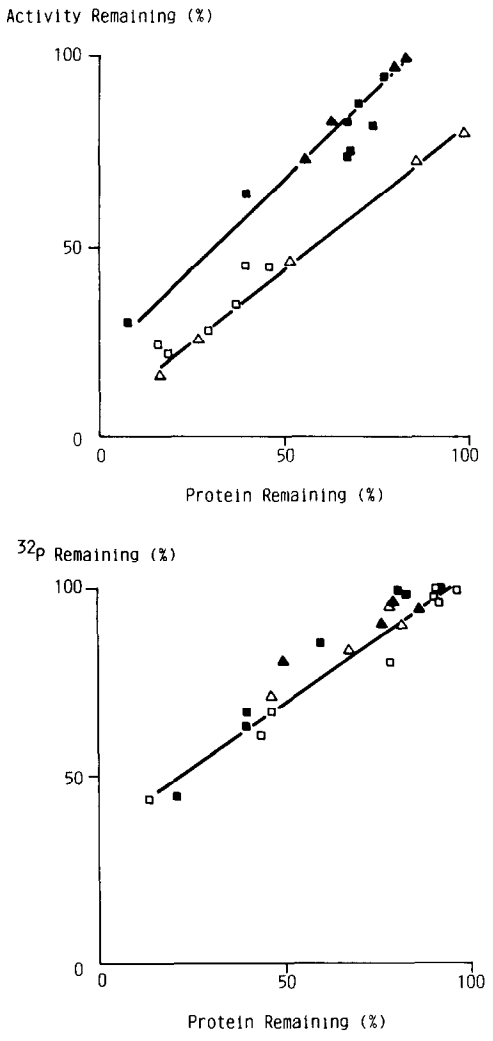

Fig.2. Correlation of loss of ICDH activity or loss of ^{32}P with loss of intact protein. Samples of ICDH were digested with chymotrypsin (□,■) or V8 protease (△,▲) in the presence (■,▲) or absence (□,△) of 0.35 mM $NADP^+$. Data are taken from tables 1,2 and two similar experiments. (a, top) Active ICDH, (b, bottom) phosphorylated ICDH.

The corresponding correlation for inactive ICDH, between release of ^{32}P and destruction of the 45-kDa component, is shown in fig.2b. The data from experiments plus or minus $NADP^+$ all fall close to a single straight line that is similar to the line observed for the active enzyme in the presence of $NADP^+$. This is in agreement with our observation that $NADP^+$ does not protect inactive ICDH against proteolysis. The results suggest that

inactive ICDH can be nicked to give a form that is not very susceptible to further attack and in which the phosphorylated region is still trichloroacetic acid-precipitable. The similarity between the results observed for inactive ICDH and active ICDH in the presence of $NADP^+$ (fig.2a,b) suggests that these two species may have a similar conformation.

In order to investigate further the interaction between ICDH and its coenzyme we studied the binding of NADPH to the enzyme fluorimetrically. Addition of NADPH to active ICDH resulted in an enhancement of the fluorescence of the coenzyme with no change in its emission and excitation spectra (not shown). The addition of NADPH to inactive ICDH had no effect on either the fluorescence yield or the excitation and emission spectra of the coenzyme. The results of fluorescence titration experiments carried out by adding NADPH to the active or inactive forms of ICDH are shown in fig.3. It is clear that NADPH binds tightly to active ICDH; the concentration of NADPH required to give saturation (4.4 μM) corresponds to a binding stoichiometry of 0.88 molecules/subunit. In contrast, NADPH appears to be unable to bind to inactive ICDH, at least at the concentrations used in this experiment.

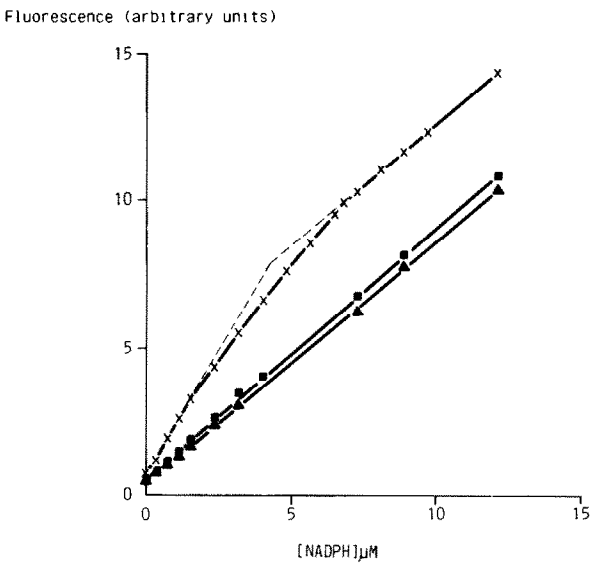

Fig.3. Fluorescence titration of ICDH with NADPH. Aliquots of NADPH solution were added to solutions of active ICDH, (×) inactive ICDH (■) or buffer alone (▲) as described in section 2. The dotted lines represent extrapolations of the two linear parts of the titration curve for active ICDH; they meet at an NADPH concentration of 4.4 μM. The ICDH concentration was 5.0 μM in terms of subunits.

4. DISCUSSION

Many of the eukaryotic enzymes regulated by phosphorylation/dephosphorylation mechanisms are allosteric proteins and phosphorylation frequently results in changes in the affinity of the enzyme for a substrate or effector rather than changes in V_{max} (e.g. [11]). There is evidence from proteolysis experiments that in many cases the phosphorylation site is not part of the active site (e.g., [12–16]). Indeed in some cases proteolytic removal of a phosphorylated peptide mimics the effects of dephosphorylation on the kinetic properties of the enzyme (e.g., [12–14]).

There are, of course, several exceptions to this general rule; e.g., mammalian pyruvate dehydrogenase (e.g., [17]). The phosphorylation of *E. coli* ICDH also does not seem to conform to the general pattern outlined above. Firstly, ICDH is not an allosteric protein; secondly, phosphorylation reduces the maximum catalytic capacity of the enzyme almost to zero. Thirdly, our results suggest that it is not possible to reactivate phosphorylated ICDH by proteolytic removal of a phosphopeptide.

Our results reveal two other interesting properties concerning the phosphorylation of ICDH. Firstly, active ICDH can be protected against proteolysis either by phosphorylation or by binding of $NADP^+$. Moreover in each case a nicked species that is relatively resistant to further attack can be produced; active iCDH in the absence of $NADP^+$ does not give rise to such a species. This suggests that phosphorylation and binding of $NADP^+$ may induce similar conformational states. Secondly, inactive ICDH seems unable to bind NADPH tightly, in contrast to the active enzyme. This result is consistent with our observation that inactive ICDH does not bind to Procion Red-Sepharose whereas active ICDH does bind and can be eluted with $NADP^+$ [9].

We have already suggested [9] that phosphorylated ICDH is inactive precisely because it cannot bind coenzyme. We now suggest further that the phosphorylation of ICDH may occur close to, or

at, the coenzyme binding site of ICDH. The hypothesis allows us to rationalise many of the properties of the phosphorylation system. The introduction of a negatively charged phosphate group on a serine residue near the NADP binding site could totally prevent coenzyme binding by charge repulsion; this in turn would explain why the phosphorylated enzyme is totally inactive. it could also trigger a conformational change similar to that induced by binding of the negatively charged coenzyme. This could explain the effects of phosphorylation and binding of $NADP^+$ on the susceptibility of ICDH to proteolysis. Our hypothesis would also explain our failure to reactivate inactive ICDH by proteolysis, a failure that is in contrast to the effects of proteolysis on enzymes such as rabbit mammary gland acetyl CoA carboxylase [13] and yeast glycogen synthase [14]. ICDH kinase is inhibited by $NADP^+$ and NADPH [7]; our hypothesis suggests that this might be caused by binding of the coenzyme to ICDH rather than to the kinase itself.

Our hypothesis offers a simple insight into how phosphorylation could inactivate an enzyme that is not allosteric; the effect may be directly at the active site. We are currently attempting to investigate this hypothesis more directly.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Science and Engineering Research Council, London. We are grateful to Barbara Brodie for excellent technical assistance.

REFERENCES

[1] Garnak, M. and Reeves, H.C. (1979) Science 203, 1111-1112.
[2] Wang, J.Y.J. and Koshland, D.E. (1981) J. Biol. Chem. 256, 4640-4648.
[3] Nimmo, H.G. (1983) Molecular Aspects of Cellular Regulation 3, in press.
[4] Laporte, D.C. and Koshland, D.E. (1982) Nature 300, 458-460.
[5] Nimmo, G.A., Borthwick, A.C., Holms, W.H. and Nimmo, H.G. (1983) submitted.
[6] Bennett, P.M. and Holms, W.H. (1975) J. Gen. Microbiol. 87, 37-51.
[7] Nimmo, G.A. and Nimmo, H.G. (1983) submitted.
[8] Laporte, D.C. and Koshland, D.E. (1983) Nature 305, 286-290.
[9] Borthwick, A.C., Holms, W.H. and Nimmo, H.G. (1983) submitted.
[10] Laemmli, U.K. (1970) Nature 227, 680-685.
[11] Cohen, P. (1983) Control of Enzyme Activity, 2nd ed., Chapman and Hall, London.
[12] Fischer, E.H., Graves, D.J., Crittenden, E.R. and Krebs, E.G. (1959) J. Biol. Chem. 234, 1698-1704.
[13] Guy, P.S. and Hardie, D.G. (1981) FEBS Lett. 132, 67-70.
[14] Huang, K.P. and Cabib, E. (1974) J. Biol. Chem. 249, 3858-3861.
[15] Takeda, Y. and Larner, J. (1975) J. Biol. Chem. 250, 8951-8956.
[16] Bergstrom, G., Ekman, P., Humble, E. and Engstrom, L. (1978) Biochim. Biophys. Acta 532, 259-267.
[17] Walsh, D.A., Cooper, R.H., Denton, R.M., Bridges, B.J. and Randle, P.J. (1976) Biochem. J. 157, 41-67.